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HPTLC method development for the simultaneous determination of Pregabalin and Amitriptyline hydrochloride in pharmaceutical dosage forms

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ABSTRACT

A simple, selective, precise high performance thin layer chromatography (HPTLC) method with densitometry at λ= 205 nm was developed and validated for simultaneous determination of Pregabalin and Amitriptyline Hydrochloride in pharmaceutical dosage form. Chromatographic separation of the drugs were performed on aluminum plates precoated with silica gel 60 F_{254} used as stationary phase and the chromatogram was developed using Toluene: Methanol: Formic acid (7: 2.5: 0.5 v/v/v) and 20 ml of mobile phase was used per chromatography run. The system was found to give a compact band for Pregabalin (R_f=0.27±0.03) and Amitriptyline Hydrochloride (R_f=0.68±0.03). The validated lowest limit of detection was 45.097ng/spot and 12.614ng/spot wherenas lowest limit of quantification was 136.659ng/spot and 38.224ng/spot for Pregabalin and Amitriptyline Hydrochloride respectively. The percentage recovery for Pregabalin was found to be 99.91 (at 50%), 99.39 (at 100%), 99.27(at 150%) and 100.42 (at 50%), 100.63 (at 100%), 100.97(at 150%) for Amitriptyline Hydrochloride. Statistical analysis proved that the method is selective, precise and accurate for the estimation of Pregabalin and Amitriptyline Hydrochloride.

Keywords: Pregabalin (PRGB), Amitriptyline Hydrochloride (AMTR), HPTLC, Pharmaceutical formulation.

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1. INTRODUCTION

Pregabalin is (*S*)-4-amino-3-(2-methylpropyl) butyric acid (Fig.1)[1 2] which is analogue of gabapentine, is more potent but very similar. It is used as anti-epileptic by binding with high affinity to the alpha-2-deltasite (subunit of calcium channels) [3]. Pregabalin also affects chemicals in the brain that send pain signals across the nervous system. Pregabalin is used to treat pain caused by fibromyalgia, or nerve pain in people with diabetes (diabetic neuropathy), herpes zoster (post-herpetic neuralgia), or spinal cord injury [4].

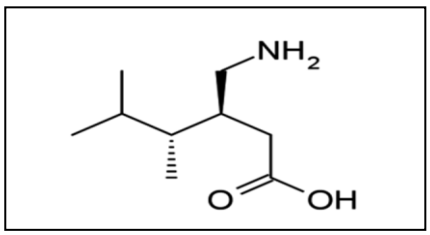

Figure 1: Pregabalin

Mol Formula: $C_8H_{17}NO_2$ **Mol Weight:** 159.229 g/mol

Amitriptyline, 3-(10, 11-dihydro-5H-dibenzo [a, d] cycloheptene-5-ylidene)-*N*, *N*-dimethyl-1-Propanamine hydrochloride (Fig.2) [2] is one of the most anticholinergic and sedative of the TCAs. Because it lacks the ring-electron–enriching nitrogen atom of imipramine, metabolic inactivation mainly proceeds not at the analogous 2-position but at the benzylic 10-position (i.e.,tolune-like metabolism predominates). Because of the 5-exocyclic double bond, E- and Z-hydroxy isomers are produced by oxidation metabolism[4]. It is tricyclic antidepressants which can elevate mood in depressive illness[5 6]. Amitriptyline increases the levels of chemical messengers in the brain that help in regulating the mood and treat depression[7].

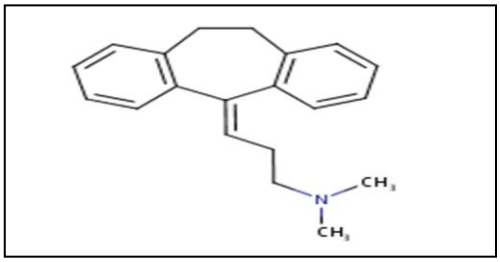

Figure 2: Amitriptyline Hydrochloride

Mol Formula: $C_{20}H_{24}ClN$ **Mol Weight:** 313.864 g/mol

2. EXPERIMENTAL

2.1 Reagents and chemicals

Pregabalin and Amitriptyline Hydrochloride were obtained as gift sample from Unichem laboratories Ltd, Pharmaceutical Company in Goa Industrial Estate, Goa, India. Toluene, Methanol, Formic acid was purchased from Merck Ltd. (Mumbai, India)

2.3 Instrumentation

CAMAG HPTLC instrument was used in this method. CAMAG HPTLC is equipped with CAMAG TLC scanner-3, Linnomate 5 Automatic sample applicator controlled by WINCATS software (1.4.2 version). Aluminum plates precoated with silica gel 60 F_{254} HPTLC plates (10 X 10cm, layer thickness 250 µm, E.MERCK)

Table 1: Optimized chromatographic conditions

Stationary phase precoated TLC plates	: Silica gel 60 F_{254}
Mobile phase	: Toluene: Methanol: Formic acid
"Mobile phase ratio (%v/v/v)	: 7.5:2:0.5
Saturation time	: 20 minutes.
Slit dimension	: 5.00 x 0.45 mm.
Source of radiation	: Deuterium.
Scan wavelength	: 205 nm.
R_f values	
Pregabalin	: 0.27±0.03.
Amitriptytline Hydrochloride	: 0.68±0.03.

2.4 Selection of analytical wavelength

From the standard stock solution further dilutions were made using methanol and scanned over the range of 200 - 400 nm and the spectra was obtained. It was observed that both the drug showed considerable absorbance at 205 nm (Fig.3)

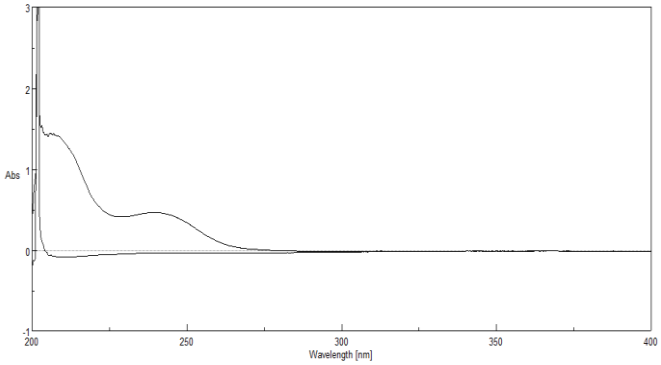

Figure 3: Overlay UV-VIS Spectra of PRGB (10 µg/ml) and AMTR (10 µg/ml)

2.5 Preparation of Standard stock solution:

Standard stock solution of PRGB and AMTR were prepared separately by dissolving 10 mg of drug in 10 ml of methanol to get concentration of 1000 µg/ml. From the respective standard stock solution, working standard solution was prepared containing 375µg/ml (375ng/µl) of PRGB and 50µg/ml (50ng/µl) of AMTR separately in methanol (Fig.4, 5, 6)

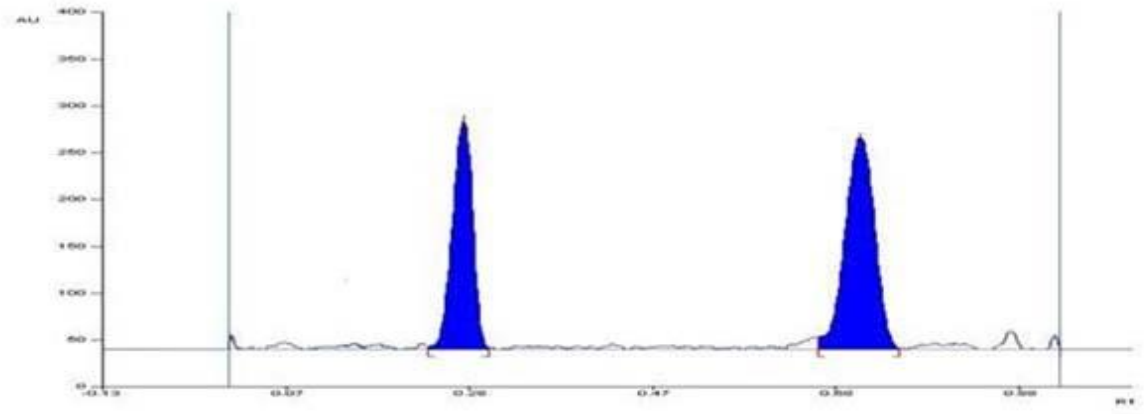

Figure 4: Densitogram of mixed standard solution of AMTR (100 ng/spot) and PRGB (750 ng/spot)

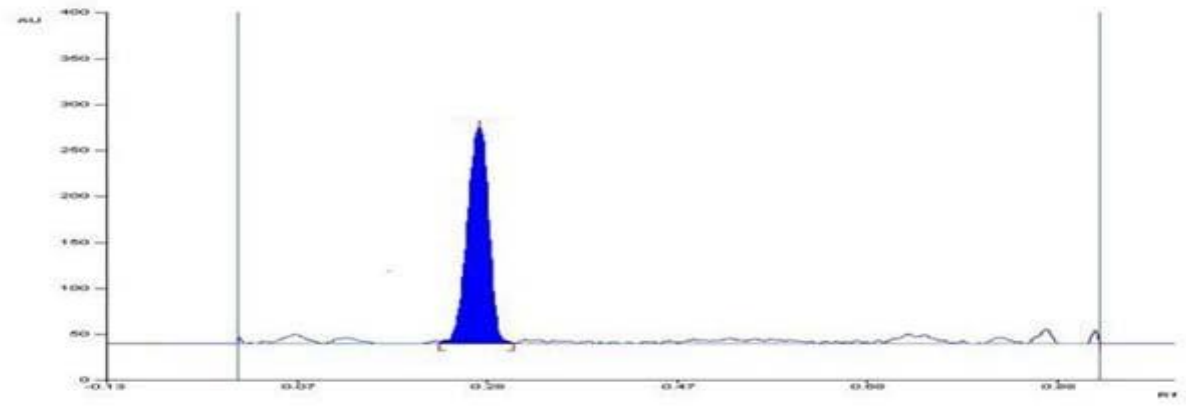

Figure 5: Densitogram of standard solution of AMTR (100 ng/spot)

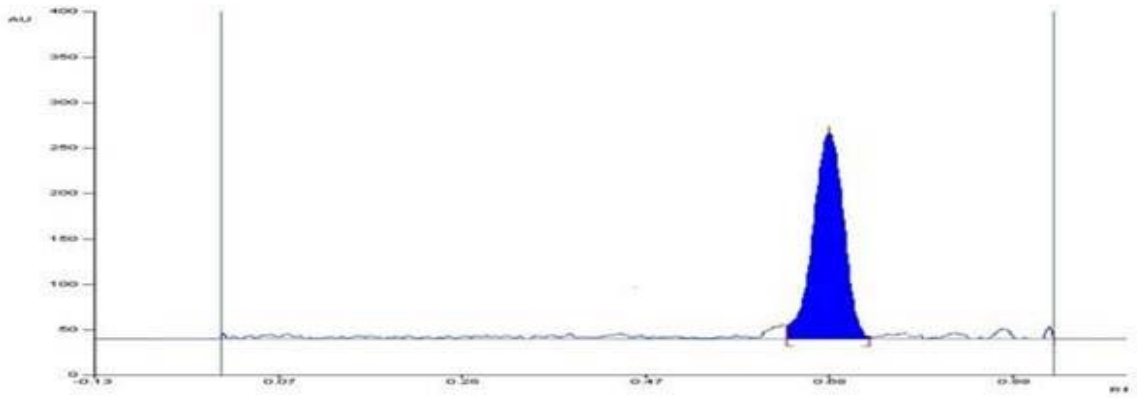

Figure 6: Densitogram of standard solution of PRGB (750 ng/spot)

2.6 Preparation of sample solution (Tablet Formulation Analysis):

Twenty tablets each containing 10 mg of AMTR and 75 mg of PRGB was weighed and powdered (Each uncoated tablet contains Pregabalin IP 75 mg and Amitriptyline HCl IP 10 mg). Powder equivalent to 10 mg of AMTR (75 mg of PRGB) was transferred to 10 ml volumetric flask and was diluted with methanol and volume made to 10 ml (1000 µg/ml of AMTR and 7500 µg/ml of PRGB) with methanol. Solution was filtered and further dilutions were made with mobile phase to get the final concentration of 50 µg/ml of AMTR and 375 µg/ml of PRGB. 2 µl volumes were applied on TLC plate to get concentration 100 ng/spot of AMTR and 750 ng/spot of PRGB (Fig.7)

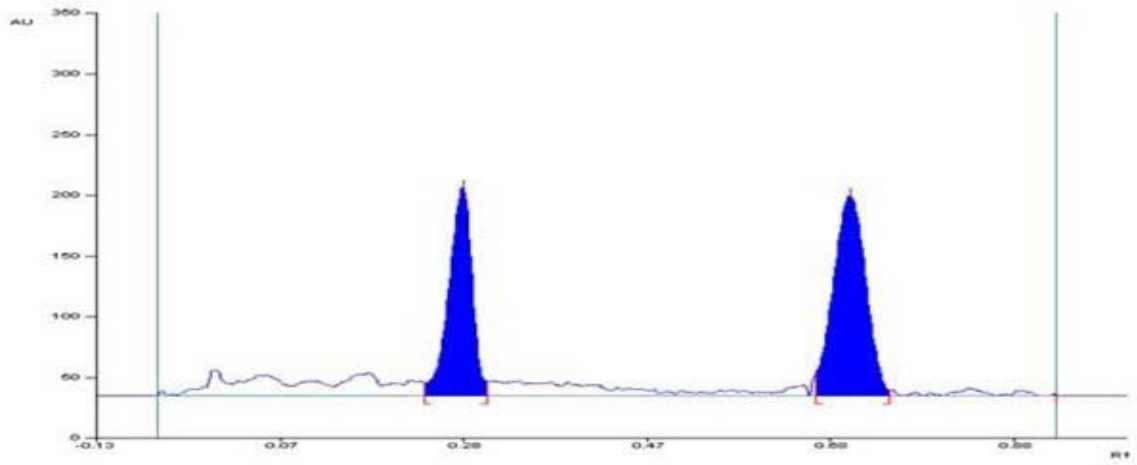

Figure 7: Densitogram of test solution (Tablet Sample) of AMTR (100 ng/spot) and PRGB (750 ng/spot)

2.7Assay

Tablet formulation analysis was carried out as mentioned under section Tablet Formulation Analysis. Procedure was repeated for six times. Sample solution was applied and area was recorded for each drug. Concentration and % purity was determined from linear equation shown in (Table 1)

Table 2: Assay results for PRGB and AMTR

Sr. no.	PRGB			AMTR		
	Peak area	Amount Recovered (ng/spot)	% Recovery	Peak area	Amount recovered (ng/spot)	% Recovery
1	4370.5	767.866	102.382	1945.5	101.728	101.728
2	4317.5	742.783	99.038	1919.4	98.856	98.856
3	4325.8	746.711	99.561	1928.7	99.879	99.879
4	4310.2	739.328	98.577	1935.2	100.594	100.594
5	4365.8	765.641	102.086	1942.8	101.430	101.430
6	4297.4	733.270	97.769	1942.5	101.397	101.397
Mean	4331.200	749.266	99.902	1935.683	100.647	100.647
% RSD	0.695	1.904	1.903	0.520	1.102	1.102

2.8 Validation of Analytical Method[8 9 10 11]

2.8.1 Linearity

From the standard stock solution (1000 µg/ml) of PRGB and AMTR, solution was prepared containing 375 µg/ml of PRGB and 10 µg/ml of AMTR separately. Different volumes were applied on TLC plate to obtain linear range. Six replicates per concentration were applied. The linearity (relationship between peak area and concentration) was determined over the concentration range 375 - 2250 ng/spot for PRGB and 50 - 300 ng/spot for AMTR. The results obtained are shown in (Table 3 and Fig.8) for PRGB and in (Table 4 and Fig.9) for AMTR.

Table 3: Linearity study of PRGB

Replicates	Concentrations of PRGB (ng/spot)					
	375	750	1125	1500	1875	2250
	Peak Area					
1	3450.20	4358.60	5200.30	5987.24	6658.23	7548.19
2	3490.50	4321.50	5218.23	5897.50	6625.49	7485.60
3	3418.56	4385.24	5190.80	5854.70	6658.23	7454.23
4	3501.00	4328.50	5240.12	5850.70	6628.90	7558.20
5	3520.40	4335.70	5279.80	5867.27	6689.20	7538.10
6	3558.20	4358.50	5290.12	5865.70	6638.90	7578.20
Mean	3489.81	4348.01	5236.56	5887.19	6649.83	7527.09
Std.dev.	49.77	23.85	41.22	51.70	23.84	47.28
%RSD	1.43	0.55	0.79	0.88	0.36	0.63

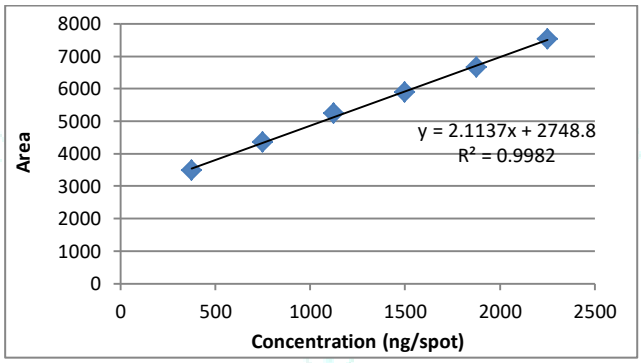

Figure 8: Calibration curve for PRGB

Table 4: Linearity study of AMTR

Replicates	Concentrations of AMTR (ng/spot)					
	50	100	150	200	250	300
	Peak Area					
1	1450.90	1980.50	2420.45	2890.70	3268.80	3695.23
2	1422.25	1995.27	2470.82	2841.68	3254.18	3715.75
3	1412.50	1950.50	2450.80	2798.92	3279.52	3652.48
4	1390.40	1917.50	2529.30	2860.70	3229.60	3749.00
5	1395.60	1929.80	2438.40	2897.40	3250.70	3784.80
6	1370.40	1919.50	2517.30	2910.50	3239.60	3759.00
Mean	1407.01	1948.85	2471.18	2866.65	3253.73	3726.04
Std.dev.	28.05	32.76	43.75	41.70	18.35	48.03
%RSD	1.99	1.68	1.77	1.45	0.56	1.29

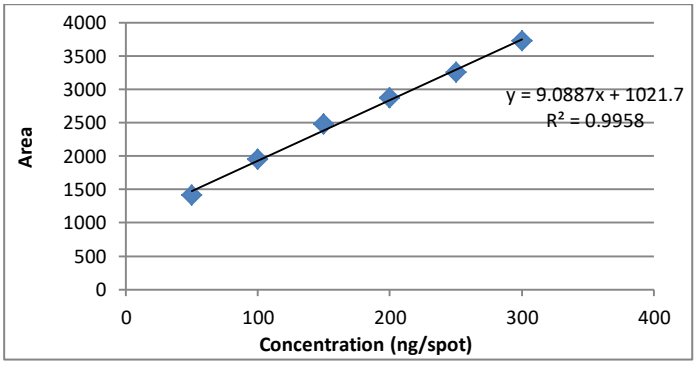

Figure 9: Calibration curve for AMTR

2.8.2 Precision:

The precision of the method was demonstrated by Intra-day and Inter-day variation studies. In the Intraday precision was found by carrying out the analysis of standard drugs at three different concentrations in the linearity range of the drugs for three times on the same day. Each concentration was applied in triplicates and % RSD was calculated. For the Inter day precision was found by carrying out the analysis of the standard drugs at three different concentrations in the linearity range of the drugs for three days and % RSD was calculated. The results obtained for Intraday and Inter day variations are shown in (Table 5, 6, 7, 8)

Table 5: Intra-day precision study of PRGB

Concentration (ng/spot)	Area	% Recovery	Avg % Recovery ± SD	Mean % Recovery ± % RSD
1125	5102.30	99.03981	99.263 ± 1.697	98.887 ± 1.222
	5070.2	97.68944		
	5150.37	101.062		
1500	5930.10	100.3975	99.344 ± 0.958	
	5889.40	99.11342		
	5870.70	98.52343		
1875	6682.90	99.31914	98.251 ± 0.965	
	6630.50	97.99653		
	6608.40	97.43871		

Table 6: Inter-day precision of PRGB

Concentration (ng/spot)	Area	% Recovery	Avg % Recovery ± SD	Mean % Recovery ± % RSD
1125	5157.3	101.354	101.107 ± 0.961	99.832 ± 1.543
	5126.23	100.046		
	5170.8	101.921		
1500	5977.24	101.885	99.968 ± 1.699	
	5897.5	99.369		
	5874.7	98.650		
1875	6658.23	98.696	98.421 ± 0.477	
	6625.49	97.870		
	6658.23	98.696		

Table 7: Intra-day precision study AMTR

Concentration (ng/spot)	Area	% Recovery	Mean % Recovery ± SD	Mean % Recovery± % RSD
150	2390.2	100.4401	99.241 ± 1.098	99.301 ± 1.008
	2370.6	99.00235		
	2360.8	98.28345		
200	2804.3	98.1129	99.574 ± 1.479	
	2830.5	99.55436		
	2857.8	101.0563		
250	3259.7	98.53433	99.087 ± 0.673	
	3289.3	99.83715		
	3267.8	98.89085		

Table 8: Inter-day precision study AMTR

Concentration (ng/spot)	Area	% Recovery	Avg % Recovery± SD	Mean% Recovery ± % RSD
150	2420.45	102.659	101.699 ± 1.159	100.764 ± 1.117
	2411.82	102.026		
	2389.8	100.411		
200	2850.7	100.666	100.834 ± 0.763	
	2841.68	100.169		
	2868.92	101.668		
250	3298.8	100.255	99.758 ± 0.547	
	3274.18	99.172		
	3289.52	99.847		

2.8.3 Accuracy

To check accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 50, 100 and 150 %. Basic concentrations of sample chosen were 2 µl of 375 µg/ml of PRGB and 2 µl of 50 µg/ml of AMTR. These solutions were applied on TLC plates in triplicate to obtain the densitogram. The drug concentrations of PRGB and AMTR were calculated by using linearity equations of PRGB and AMTR. The results obtained are shown in (Table 9, 10)

Table 9: Recovery studies of PRGB

Level	Conc. (ng/spot)		Area	% Recovery	Mean % Recovery ± RSD
	Sample	Std.			
50 %	750	375	5130.5	100.226	99.909 ± 0.468
			5128.2	100.129	
			5110.2	99.372	
100 %	750	750	5910	99.763	99.387 ± 0.336
			5889.8	99.126	
			5894.4	99.271	
150 %	750	1125	6695.8	99.645	99.273 ± 0.478
			6687.5	99.435	
			6659.9	98.739	

Table 10: Recovery studies of AMTR

Level	Conc. (ng/spot)		Area	% Recovery	Mean % Recovery ± RSD
	Sample	Std.			
50 %	100	50	2398.5	101.049	100.418 ± 0.621
			2389.7	100.403	
			2381.5	99.802	
100 %	100	100	2855.4	100.924	100.627 ± 0.269
			2845.8	100.396	
			2848.8	100.561	
150 %	100	150	3314	100.924	100.973 ± 0.488
			3326.8	101.488	
			3304.5	100.506	

2.8.4 Limit of detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S respectively. Where (σ) is the standard deviation of the response (y-intercept) and (S) is the mean of the slop of calibration plot. The LOD values of PRGB and AMTR was found to be 45.097ng/spot and 12.614ng/spot respectively and the LOQ values were found to be 136.659ng/spot and 38.224ng/spot.

2.8.5 Robustness:

Robustness of the method was determined by carrying out the analysis under conditions during which wavelength, chamber saturation time and time form application to development was altered and the effect on area was noted. The results obtained are shown in (Table 11).

Table 11: Robustness study

Drug	% RSD Found for Robustness Study (peak area)								
	Wavelength			Chamber Saturation Time (Min)			Time form application to development		
	204	205	206	19	20	21	0	30	60
PRGB	1.011	0.562	0.646	0.794	0.647	0.775	0.806	0.584	1.088
AMTR	0.858	0.756	1.106	1.796	1.245	1.857	1.755	1.045	0.878

3. RESULT AND DISSCUSSION

This study was aimed at the development of sensitive, economical and less time consuming HPTLC technique for the determination of Pregabalin and Amitriptyline Hydrochloride in pharmaceutical dosage form. Well resolved by the relevant ICH guidelines and other current regulatory guidelines. The chromatographic conditions were optimized to achieve the best resolution and peak shape for Pregabalin and Amitriptyline Hydrochloride. UV scanning at 200-400 nm for both Pregabalin and Amitriptyline Hydrochloride show that 205 nm is the suitable wavelength for detection of drug (Fig.3). Different mobile phase in different proportion were tried and the mobile phase containing Toluene: Methanol: Formic acid (7: 2.5: 0.5 v/v/v) was selected as optimal for obtaining well resolved peaks of Pregabalin (R_f

=0.27±0.03) and Amitriptyline Hydrochloride (R_f =0.68±0.03) with acceptable system suitability parameters (Fig.4). The linearity (relationship between peak area and concentration) was determined over the concentration range 375 - 2250 ng/spot (r^2 =0.999) for Pregabalin (Table. 3 and Fig. 8) and 50 - 300 ng/spot (r^2 =0.999) for Amitriptyline Hydrochloride (Table. 4 and Fig. 9). The LOD and LOQ were found to be 45.097, 12.614ng/spot, 136.659, 38.224ng/spot respectively for Pregabalin and Amitriptyline Hydrochloride. The method was found to be precise based on the results obtained in the intraday and inter-day precision evaluation study. These results were expressed in terms of % RSD that was found to be less than 2 (Table 5, 6, 7, 8). High recovery values followed by low % RSD value (<2) coupled with low standard deviation makes the proposed method highly suitable for accurate and precise determination of Pregabalin and Amitriptyline Hydrochloride in combined tablet dosage forms. Closeness of the amount found to the amount taken and low coefficient of variation value showed that the proposed method was accurate and precise. Recovery study conducted by HPTLC method was performed by spiking 50, 100 and 150 % of additional drug recovery of 99.91-99.39 % for Pregabalin and 100.42-100.97 % for Amitriptyline Hydrochloride as listed in (Table 9 and 10). To evaluate the robustness of the method, the parameters selected were varied at three levels. The results indicate that less variability in retection time were observed. (Table 11)

4. CONCLUSION

Introducing HPTLC into pharmaceutical analysis represents a major step in terms of quality assurance. The developed HPTLC technique is precise, specific and accurate. Statistical analysis proves that the method is suitable for the analysis of Pregabalin and Amitriptyline Hydrochloride as bulk drug and in pharmaceutical formulation without any interference from the excipients. It was concluded that the developed method offered several advantages such as rapid, cost effective, simple mobile phase and sample preparation steps and improved sensitivity made it specific, reliable and easily reproducible in any quality control setup providing all the parameters are followed accurately for its intended use.

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